Stimulation of All Epithelial Elements during Skin Regeneration by Keratinocyte Growth Factor

By Glenn F. Pierce,* Donna Yanagihara,* Kathleen Klopchin,* Dimitry M. Danilenko,* Eric Hsu,‡ William C. Kenney,‡ and Charles F. Morris§

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Summary

Keratinocyte growth factor (KGF), a recently discovered 18.9 kD member of the fibroblast growth factor family has been shown to selectively induce keratinocyte proliferation and differentiation in tissue culture. To explore its potential stimulating keratinocyte growth and differentiation in vivo, we analyzed for the influence of KGF on epithelial derived elements within a wound created through the cartilage on the rabbit ear. KGF accelerated reepithelialization ($p = 0.004$) and increased the thickness of the epithelium ($p = 0.0005$) when 4–40 $\mu g/cm^2$ recombinant KGF was added at the time of wounding. The regenerating epidermis showed normal differentiation as detected by cytokeratin immunostaining. Remarkably, however, KGF stimulated proliferation and differentiation of early progenitor cells within hair follicles and sebaceous glands in the wound bed and adjacent dermis. There was a transient but highly significant increase in specific labeling of cycling cells in both basal and suprabasal layers that extended into the spinous layer of the regenerating epidermis. As an indication of specificity, the inflammatory cells and fibroblasts within the wound were not influenced by KGF. The results indicate that KGF is unique in its ability to accelerate reepithelialization and dermal regeneration by targeting multiple epithelial elements within the skin. These results suggest that KGF may induce specific epithelial progenitor cell lineages within the skin to proliferate and differentiate, and thus may be a critical determinant of regeneration of skin. Furthermore, these findings illustrate the potential capacity of this system to analyze epithelial differentiation programs and disorders of epidermis, dermal glandular elements, and hair follicles.

Keratinocyte growth factor (KGF[1]; fibroblast growth factor [FGF] 7) was originally purified as a mitogenic activity for keratinocytes from an embryonic lung fibroblast line (1). KGF was found to be ~ 30–45% homologous to the other seven members of the FGF family (2). Unlike acidic and basic FGFs, KGF contains a putative signal sequence. KGF interacts with a specific splice variant of the *bek* (FGFR2) receptor, a receptor that is similar to *K-sam*, and does not bind to *flg* (FGFR1) or its splice variants (3–5). Although acidic FGF also binds the KGF receptor with similar affinity as KGF, acidic FGF binds other FGF receptors as well, and thus acidic and basic FGFs have a greater target cell range (e.g., endothelial cells, fibroblasts) than KGF, which is only known to stimulate epithelial cells (1, 2, 6). Basic FGF does not bind the KGF receptor, but like acidic FGF, does bind FGFR2. Within the KGF receptor, 49 amino acids are alternatively spliced into the COOH-terminal portion of the third Ig loop, which confers binding specificity for KGF (7–9).

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After 0.25 cm^2 wounds were created with a 6-mm trephine, rKGF at specified concentrations or vehicle alone (PBS) was applied once on the day of surgery, and the wounds were covered with Tegaderm occlusive dressing (3M, St. Paul, MN) (7). Wounds were harvested from 1 to 7 d after wounding. At sacrifice, each wound was bisected; one section was frozen in optimum cooling temperature medium (Miles, Inc., Elkhart, IN) and the second was fixed in Omnifix II (Al-Con Genetics, Inc., Melville, NY) and processed according to routine histological methods. Masson Trichrome, oil red o, and immunohistochemical stains were performed on 3 μm–thin sections for each wound.

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Country Application

Thursday, January 29, 2004

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European Patent Convention
Case Type: PCT
Lic.: HGS **CPI Case:** ☑
Application Status: Published
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Filing Date: 01-Jun-2000
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Agent: VP
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Agent Reference No.: F 2998 EP S3
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Tax Schedule: LE
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National Stage Date:

List Of Actions

Action(s) Due	Due Date		Action Taken
Annuity Due Reminder	01-Apr-2004	Reminder	
1 Month Until Response Due Rem	05-May-2004	Reminder	
Annuity Due Final	01-Jun-2004	Final	
Response Due	05-Jun-2004	Due Date	
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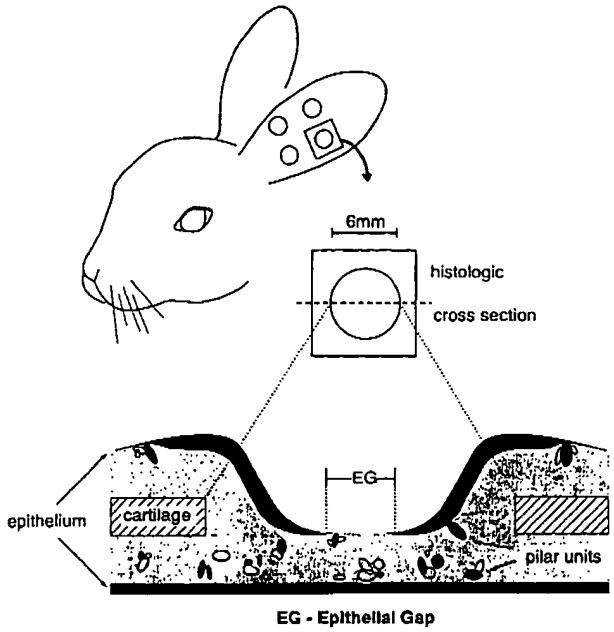


Figure 1. Modified rabbit ear partial thickness dermal wound model. The rabbit ear dermal ulcer model (11, 12) was modified to produce a wound through the cartilage, to the dermis on the back side of the ear.

significant increase in reepithelialization occurred when 1 μg rKGF was added to wounds (76.9 ± 5.8% rKGF vs. 52.5 ± 6.3%, controls; $p = 0.004$). The thickness of the new epithelium covering the wound also was markedly increased in a dose-dependent fashion at both 5 and 7 d after wounding to nearly twice the area of control wound epithelium at a dose of up to 10 μg per wound (40 μg/cm^2; Fig. 2).

Importantly, histologic analysis suggested that enhanced epithelial regeneration in rKGF-treated wounds occurred via migration of outer root sheath keratinocytes within the underlying dermal wound bed as well as from wound borders (Fig. 3), suggesting that rKGF could influence epithelial cells within the wound bed as well. These observations are consistent with the known phenotypic plasticity of epithelial cells within pilosebaceous units which permits them to develop into epidermis in vitro, as well as in vivo, within partial thickness wounds (16). Furthermore, enhanced reepithelialization in the wounds exposed to underlying dermis, but not in full thickness wounds, suggests that adnexal elements are critical targets of rKGF. Granulation tissue formation was not enhanced (Fig. 3), as assessed by quantitative image analysis of wound areas and volumes, suggesting that rKGF had a specificity distinct from platelet-derived growth factor or basic FGF, growth factors that can also activate fibroblasts and endothelial cells and enhance matrix deposition (12, 17, 18).

Because rKGF greatly enhanced epidermal regeneration, we sought to determine whether it also enhanced epidermal maturation as revealed by immunostaining for cytokeratins 14 and 10. In unwounded epidermis, cytokeratins 14 and 10 are found within basal and suprabasal keratinocytes, respectively. Immunostaining of regenerating epidermis revealed that rKGF did not accelerate terminal differentiation of keratinocytes, as assessed by lack of cytokeratin 10 expression in both rKGF-treated and control wounds (Fig. 4). However, rKGF greatly increased a normal population of less mature cytokeratin 14 positive cells that had migrated into the wound bed (Fig. 4). 5-d-old rKGF-treated wounds also appeared more intensely stained for cytokeratin 14 than did control wounds, suggesting that cytokeratin 14 expression may be upregulated in rKGF-treated wounds. These data indicated that rKGF does not alter two differentiation markers within regenerating epidermis, but augments the number of immature keratinocytes present.

Since rKGF enhanced epidermal regeneration but not maturation, we next sought to quantify and localize keratinocyte proliferation within the regenerating epidermis using 5-BrdU, an S-phase marker (13). Rabbits were injected with BrdU for 30–60 min before harvest, and wounds were analyzed 24, 30, 48, and 120 h after wounding. Only minimal (baseline) epidermal proliferation was observed at 24 h. After 30 h, markedly increased numbers of proliferating basal keratinocytes at the wound margins were detected in rKGF-treated wounds, suggesting that they were stimulated directly by

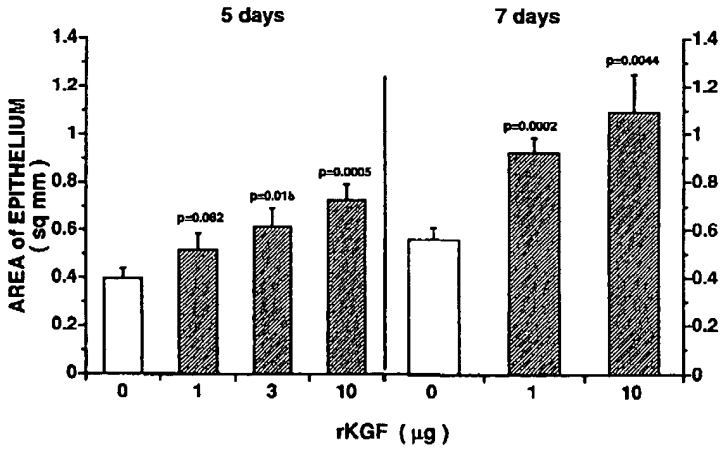


Figure 2. Total area of regenerating epithelium at days 5 and 7 after wounding in rKGF-treated and control wounds.

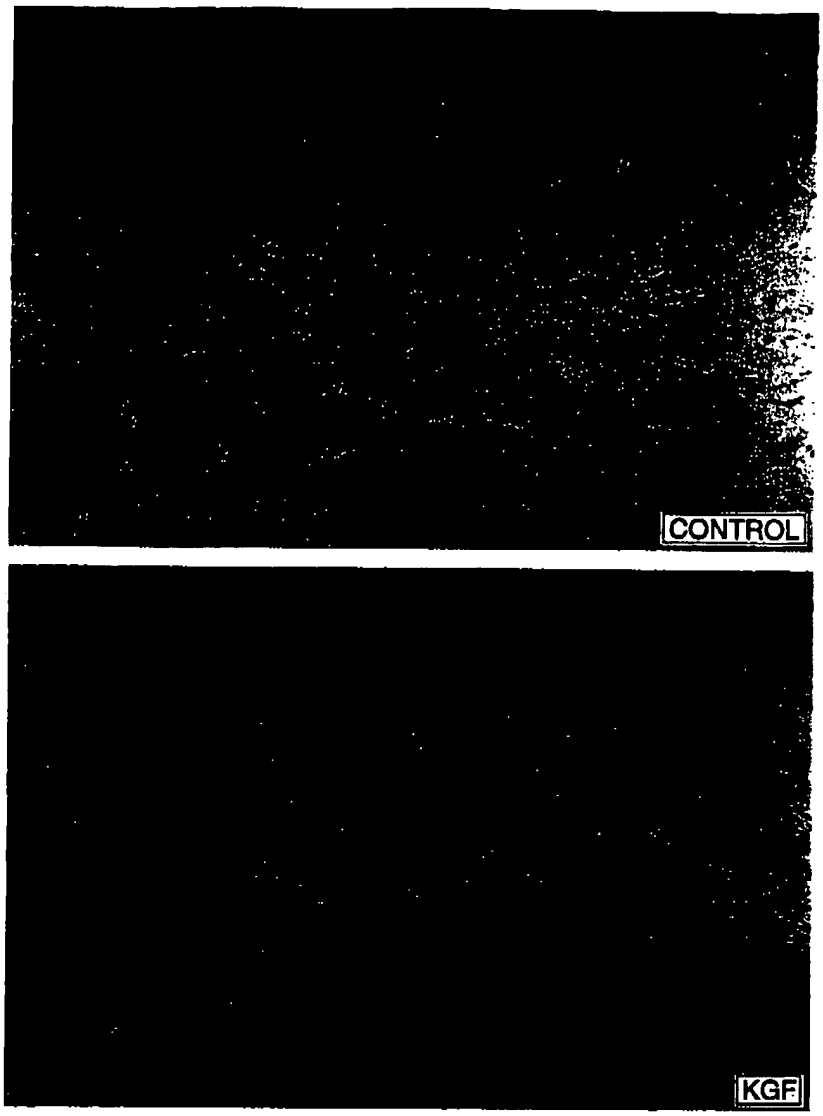


Figure 3. Proliferation and differentiation of keratinocytes. Histologic analysis of 10 μg rKGF-treated and control wounds showing increased keratinocyte proliferation and no increased granulation tissue in rKGF-treated wounds (×160, BrdU and hematoxylin counterstain). Note increased proliferation of peripheral sebocyte stem cells in the rKGF-treated wound.

rKGF to enter the cell cycle ($p = 0.05$; Fig. 5). In addition, nearly twice as many proliferating suprabasal keratinocytes were detected in rKGF-treated wounds ($p = 0.02$). By days 2 and 5, increased numbers of proliferating keratinocytes in rKGF-treated wounds were detected primarily in the suprabasal layer of the cytokeratin 14 positive neoepidermis, suggesting continued proliferation of undifferentiated phenotypically basal keratinocytes (Fig. 6). The transient burst of basal keratinocyte proliferation detected at 30 h in rKGF-treated wounds indicates self-limited acceleration of epithelial repair and differentiation.

To better analyze migration of keratinocytes as a contributor to the accelerated repair observed with rKGF, explants of rabbit skin were cultured for 4 d in suspension. They were analyzed for the potential of the epithelium to undergo epiboly, and to fully epithelialize the bottom dermal surface of the explant in response to rKGF. A dose-dependent increase in epithelial migration around the exposed dermal collagen was observed with maximal effects at 160 ng/ml (Table 1).

Of particular note, both hair follicles and sebaceous glands were considerably larger and more numerous in rKGF-treated wounds compared with control wounds. However, because the size of the pilosebaceous units was difficult to precisely quantify in tissue sections because of their asynchronous growth and cellular content, wounds from BrdU-injected rabbits were assessed for the extent of sebocyte and hair follicular cell proliferation in response to rKGF. A dose-dependent increase in the number of follicles, and proliferating cells per follicle was observed in rKGF-treated wound beds (Table 2). Furthermore, increased numbers of proliferating follicles per wound were observed, and total numbers of proliferating follicular cells were increased in rKGF-treated wounds (Table 2, Fig. 7). Proliferation was not confined to the bulge region, where the stem cells are thought to reside (19), but

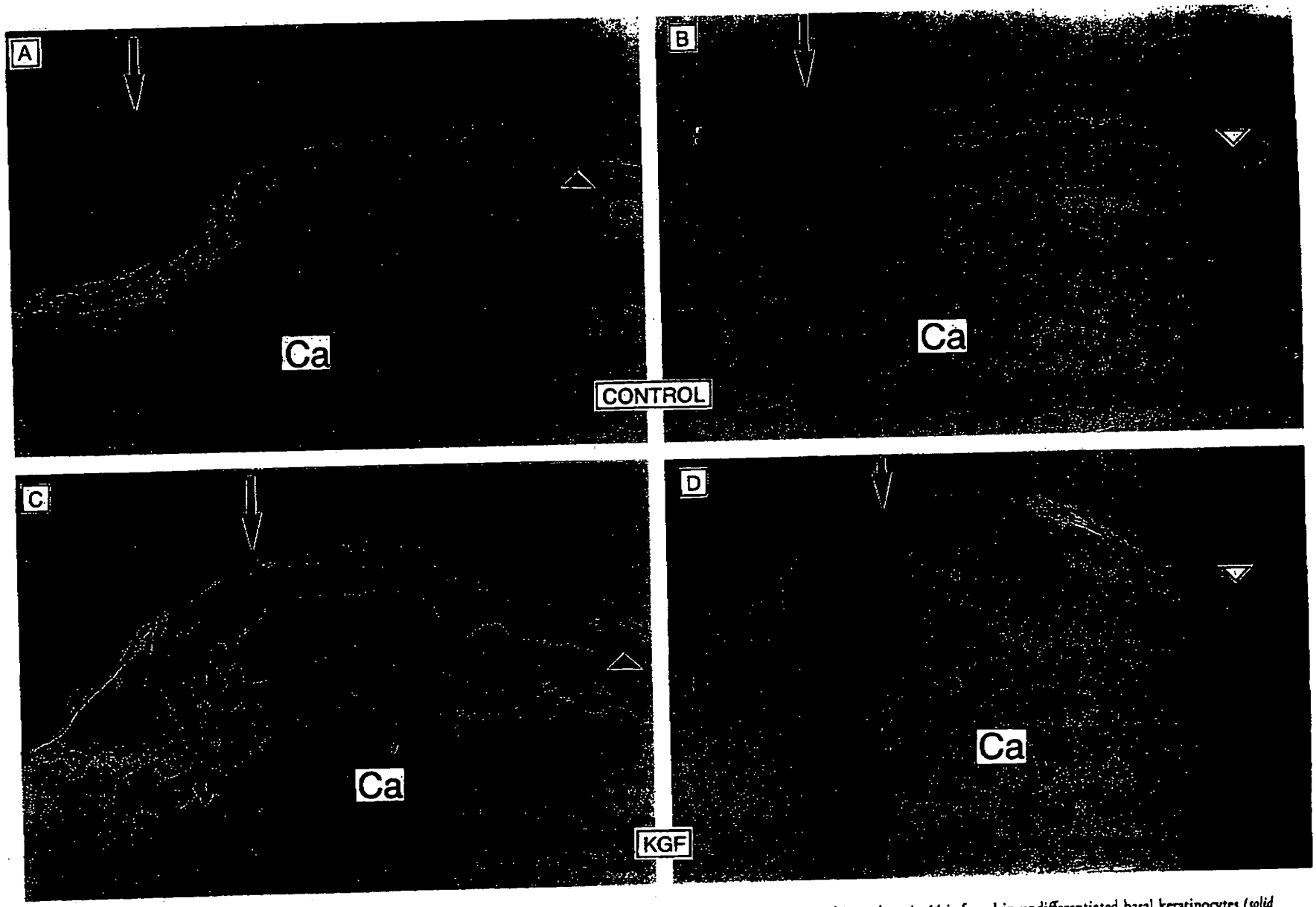


Figure 4. Cytokeratin 14 and cytokeratin 10 immunostaining of regenerating epidermis. In normal skin (right edges of panels) cytokeratin 14 is found in undifferentiated basal keratinocytes (*solid arrowheads*) and cytokeratin 10 is found in differentiating suprabasal keratinocytes (*open arrowheads*). 5-d-old rKGF-treated and control wounds were stained with antibodies to cytokeratins 14 (*A*, control; *C*, rKGF) and 10 (*B*, control; *D*, rKGF). (*Arrows*) Original wound margin; (*Ca*) cartilage.

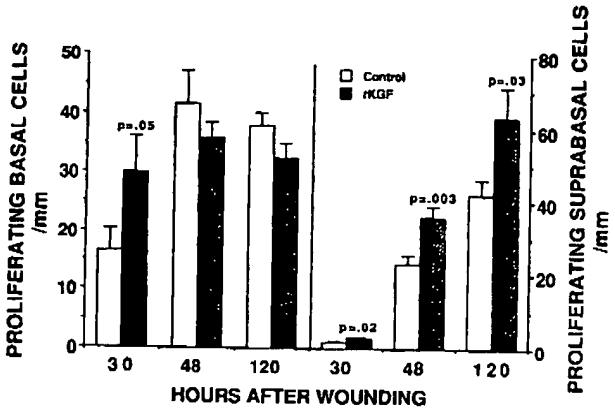


Figure 5. Proliferation of basal (*left*) and suprabasal (*right*) keratinocytes. BrdU was administered within 1 h of sacrifice, thus proliferation, and not migration of proliferating cells, is being measured.

Table 1. *Relative Amount of New Epithelium in Cultured Skin Explants*

rKGF dose	rKGF> paired control
ng/ml	
2.6	3/6
10	3/6
40	4/6
160	6/6*

Normal skin biopsies from the rabbit ear were bisected. One half was treated with rKGF, the other half served as control. After 4 d of culture, cross sections of each biopsy half were evaluated histologically for the extent of new epithelium present on the dermal surface (epithelialization). $n = 6$ per dose group.
* $p = 0.014$, Wilcoxon signed rank test.

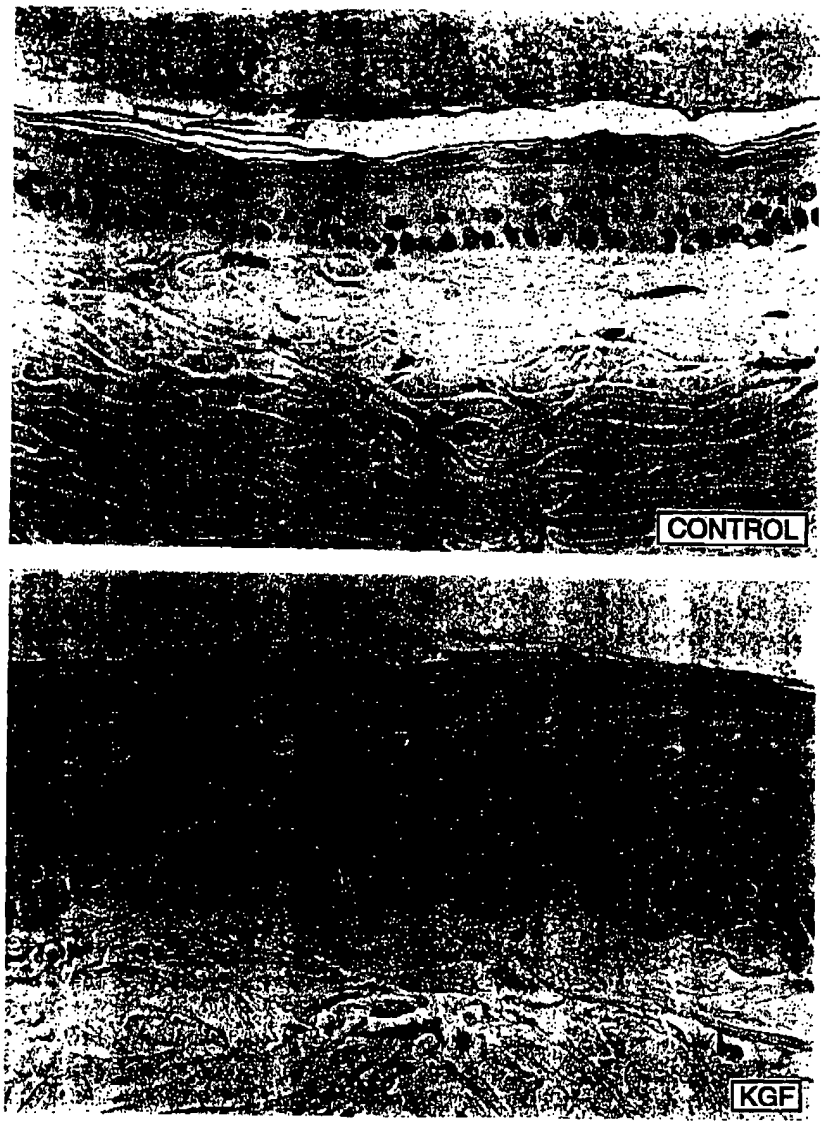


Figure 6. Proliferating basal and suprabasal keratinocytes per millimeter regenerating epithelial tongue 2 d after wounding in 3 μg rKGF-treated and untreated wounds (×320, BrdU and hematoxylin counterstain). Note increased suprabasal keratinocyte proliferation in the rKGF-treated wound.

Table 2. *Proliferating Hair Follicle Epithelial Cells and Sebocytes in rKGF-treated Wounds*

	rKGF (μg)				
	0	1	3	10	*P* value
Follicles/wound bed	2.0 ± 0.3	3.8 ± 1.7	6.0 ± 2.3	4.1 ± 1.1	NS
Proliferating cells/ follicle	19.4 ± 5.2	13.8 ± 2.2	29.2 ± 3.6	33.2 ± 4.3*	0.04*
Proliferating follicle cells/wound bed	33 ± 2	53 ± 4	175 ± 8	138 ± 5	NS
Percent wounds with proliferating cells	30	29	67	88	<0.05‡
Glands/wound bed	15 ± 2	22 ± 3	31 ± 3	27 ± 4	<0.01§
Proliferating glandular cells/wound bed	6 ± 3	ND	ND	65 ± 19+	<0.01§

5 d wounds from rabbits treated with rKGF or left untreated were stained for proliferating cells using anti-BrdU immunohistochemistry. The number of proliferating cells within each follicle, and within all follicles in the entire wound bed was determined by one individual blinded to the treatments. Because of their asynchronous growth, only follicles containing five or more proliferating cells were used for the analyses. At least 7 wounds were analyzed for each dose group. Mean ± SE are presented.

* Kruskal-Wallis test.

‡ Chi square test.

§ One-way analysis of variance, Dunnett *t* test.

also was observed throughout the hair bulb in the outer root sheath. Furthermore, in preliminary experiments, rKGF greatly reduces hair loss in a chemotherapy-induced alopecia model we have established in rats (Yanagihara, D., and G. F. Pierce, unpublished observations). Taken together, these results suggest that rKGF may potentiate the anagen, or growth phase of hair follicles, although whether hair production is increased is not yet known.

Sebocyte maturation consists of a series of steps in which peripheral undifferentiated cells proliferate and subsequently migrate inward into the lobules of glands where they gradually differentiate into mature sebum-producing cells (20, 21). The number of sebaceous glands, and the number of proliferating sebocytes, was significantly increased within rKGF-treated wounds as seen in Fig. 3 and Table 2. To more selectively identify differentiation within sebaceous glands, sections were stained with oil red o. The results demonstrated that glands were markedly hyperplastic in rKGF-treated wounds, indicating that rKGF enhances proliferation and differentiation of sebocytes into sebum-producing cells (Fig. 7 *b*). The influence of rKGF on adnexae was not confined to the wound bed and underlying dermis, but was also observed in the dermis above the cartilage adjacent to the wounds.

Both epidermal growth factor (EGF) and basic FGF have been examined in a similar model of wound healing in the rabbit ear (11, 12). Although both EGF and basic FGF can stimulate reepithelialization, striking differences when compared with rKGF were found. Neither EGF nor basic FGF influenced proliferation or differentiation of adnexal structures (11). In fact, EGF induces catagen regression and cell death within hair follicles and sebaceous glands (22–25, and G. F. Pierce, unpublished observations), and basic FGF inhibits development of the pilosebaceous units in newborn mice (26).

These results thus indicate that rKGF is uniquely capable of directly stimulating multiple epithelial stem cells in the skin. The increased proliferation in follicles and sebaceous glands coupled with enhanced reepithelialization suggest that rKGF may directly stimulate putative progenitor cells within pilosebaceous units as well as more mature keratinocytes located in the outer root sheath (16). In support of this observation, Guo et al. (27) recently expressed KGF in basal keratinocytes of mice using the cytokeratin 14 promoter, and found diminished hair follicle morphogenesis from a multipotential epithelial cell precursor in the fetus. Taken together, both studies (this report and 27) support the notion that KGF can alter differentiation pathways within pilosebaceous units in embryogenesis and wound healing, and suggest that KGF may be important in elucidating the differentiation programs of epithelial stem cells.

An initial burst of basal keratinocyte proliferation coupled with more sustained proliferation of emerging suprabasal cells and migration of regenerating basal keratinocytes is also likely an important contributor to the enhanced reepithelialization mediated by rKGF in this modified deep partial thickness model. Additional markers of differentiating keratinocytes will be important in defining the relative contributions of pilosebaceous units and bordering basal keratinocytes toward wound

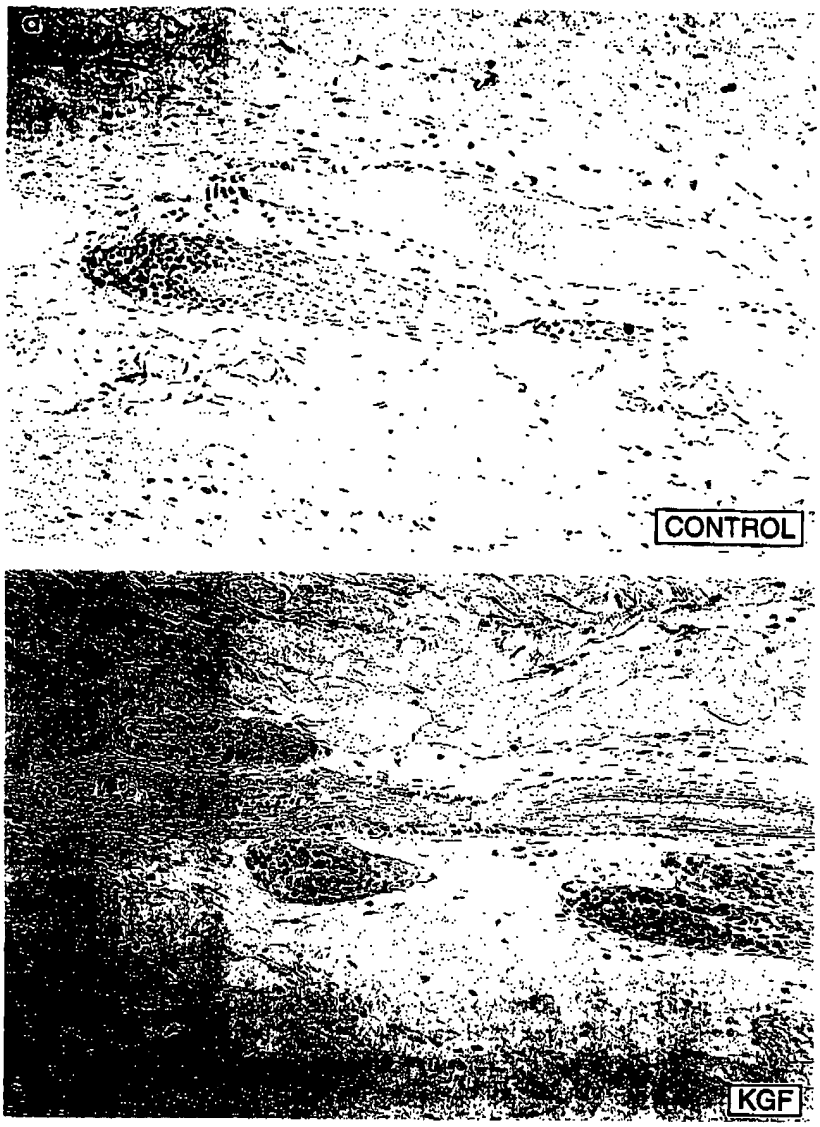
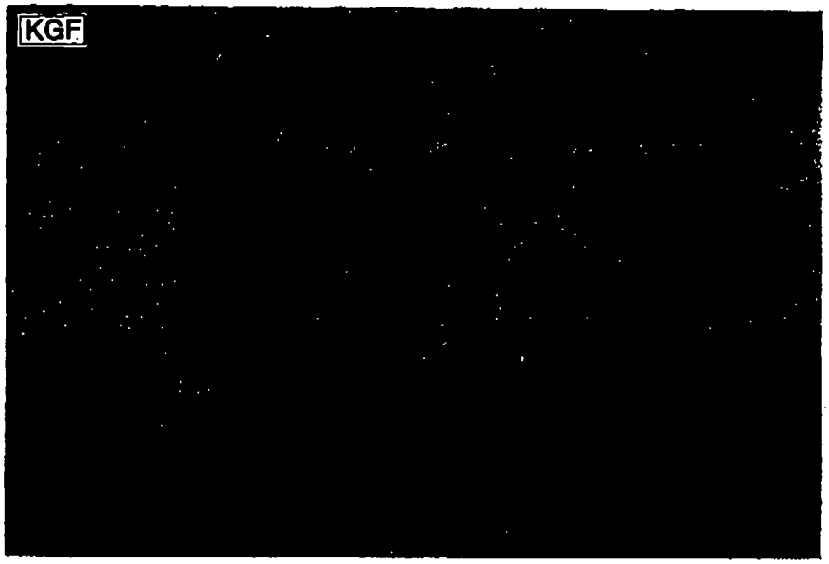


Figure 7. Influence of KGF on dermal adnexae. (*a*) Histologic analysis of proliferating hair follicles within 1-d-old wounds treated with 3 μg rKGF or untreated (×160, BrdU and hematoxylin counterstain). Note increased proliferation within the shaft of the follicle, including the outer root sheath and bulge regions (19). (*b*) Oil red o staining of sebaceous glands treated with 10 μg rKGF or untreated at the base of 5-d-old wounds (×160, hematoxylin counterstain).

reepithelialization in rKGF-treated wounds (28–30). It also would be of interest to determine if rKGF augments or accelerates expression of specific integrin subunits in keratinocytes from healing wounds, since enhanced expression of suprabasal integrins α2, α3, α6, and β1 has been observed in hyperproliferative epidermis and may play a role in migration, adhesion, and terminal differentiation of keratinocytes in wounds (31, 32).

The ability to stimulate proliferation and subsequent differentiation of multiple epithelial cell types within the skin, coupled with its original isolation from fibroblasts, suggest that KGF is a potent paracrine stimulator of the skin regenerative process. In support of this hypothesis, Werner et al. (10) recently observed marked and rapid induction of KGF mRNA in the healing dermis of mouse partial thickness wounds. In porcine partial thickness wounding models, rKGF also was shown to enhance reepithelialization (33 and G. F. Pierce, unpublished), and to accelerate maturation of the epidermal–dermal junction in healed wounds (33). Our results therefore suggest that KGF has a unique target cell spectrum compared with the other FGF family members and EGF-like growth factors (34), and may be of therapeutic value in diseases of, or injury to, skin, in which full regeneration is needed.



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References

1. Rubin, J.S., H. Osada, P.W. Finch, W.G. Taylor, S. Rudikoff, and S.A. Aaronson. 1989. Purification and characterization of a newly identified growth factor specific for epithelial cells. *Proc. Natl. Acad. Sci. USA.* 86:802.
2. Finch, P.W., J.S. Rubin, T. Miki, D. Ron, and S.A. Aaronson. 1989. Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. *Science (Wash. DC).* 245:752.
3. Bottaro, D.P., J.S. Rubin, D. Fon, P.W. Finch, C. Florio, T. Miki, D. Ron, and S.A. Aaronson. 1990. Characterization of

the receptor for keratinocyte growth factor: evidence for multiple fibroblast growth factor receptors. *J. Biol. Chem.* 265:12767.

4. Miki, T., T. Fleming, D. Bottaro, J. Rubin, D. Ron, and S.A. Aaronson. 1991. Expression cDNA cloning of the KGF receptor by creation of a transforming autocrine loop. *Science (Wash. DC).* 251:72.
5. Miki, T., D.P. Bottaro, T.P. Fleming, C.L. Smith, W.H. Burgess, A.M.-L. Chan, and S.A. Aaronson. 1992. Determination of ligand-binding specificity by alternative splicing: two distinct growth factor receptors encoded by a single gene. *Proc. Natl. Acad. Sci. USA.* 89:246.
6. Marchese, C., J. Rubin, D. Ron, A. Faggioni, M.R. Torrisi, A. Messina, L. Frati, and S.A. Aaronson. 1990. Human keratinocyte growth factor activity on proliferation and differentiation of human keratinocytes: differentiation response distinguishes KGF from EGF family. *J. Cell. Physiol.* 144:326.
7. Dell, K.R., and L.W. Williams. 1992. A novel form of fibroblast growth factor receptor 2. *J. Biol. Chem.* 267:21225.
8. Yayon, A., Y. Zimmer, S. Guo-Hong, A. Avivi, Y. Yarden, and D. Givol. 1992. A confined variable region confers ligand specificity on fibroblast growth factor receptors: implications for the origin of the immunoglobulin fold. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:1885.
9. Bottaro, D.P., E. Fortney, J.S. Rubin, and S.A. Aaronson. 1993. A keratinocyte growth factor receptor–derived peptide antagonist identifies part of the ligand binding site. *J. Biol. Chem.* 268:9180.
10. Werner, S., K.G. Peters, M.T. Longaker, F. Fuller-Pace, M.J. Banda, and L.T. Williams. 1992. Large induction of keratinocyte growth factor expression in the dermis during wound healing. *Proc. Natl. Acad. Sci. USA.* 89:6896.
11. Mustoe, T.A., G.F. Pierce, C. Morishima, and T.F. Deuel. 1991. Growth factor induced acceleration of tissue repair through direct and inductive activities. *J. Clin. Invest.* 87:694.
12. Pierce, G.F., J. Tarpley, D. Yanagihara, T.A. Mustoe, G.M. Fox, and A. Thomason. 1992. PDGF-BB, TGF-β1, and basic FGF in dermal wound healing: neovessel and matrix formation and cessation of repair. *Am. J. Pathol.* 140:1375.
13. Gratzner, H.G. 1982. Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: a new reagent for detection of DNA replication. *Science (Wash. DC).* 218:474.
14. Potten, C.S., and R.J. Morris. 1988. Epithelial stem cells *in vivo*. *J. Cell Sci. Suppl.* 10:45.
15. Dover, R., and F.W. Watt. 1987. Measurement of the rate of epidermal terminal differentiation: expression of involucrin by S-phase keratinocytes in culture and in psoriatic plaques. *J. Invest. Dermatol.* 89:349.
16. Lenoir, M.-C., B.A. Bernard, G. Pautrat, M. Darmon, and B. Shroot. 1988. Outer root sheath cells of human hair follicle are able to regenerate a fully differentiated epidermis *in vitro*. *Dev. Biol.* 130:610.
17. Pierce, G.F., T.A. Mustoe, R.M. Senior, J. Reed, G.L. Griffin, A. Thomason, and T.F. Deuel. 1988. In vivo incisional wound healing augmented by platelet-derived growth factor and recombinant c-*sis* gene homodimeric proteins. *J. Exp. Med.* 167:974.
18. Pierce, G.F., J. Vande Berg, R. Rudolph, K. Doria, J. Tarpley, and T.A. Mustoe. 1991. Platelet derived growth factor-BB and transforming growth factor-β1 differentially augment inflammatory and matrix assembly phases of wound healing and inhibit myofibroblast generation: ultrastructural and morphometric analyses. *Am. J. Pathol.* 138:629.
19. Cotsarelis, G., T.-T. Sun, and R.M. Lavker. 1990. Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell.* 61:1329.
20. Latham, J.A.E., C.P.F. Redfern, A.J. Thody, and T.A. De Kretser. 1989. Immunohistochemical markers of human sebaceous gland differentiation. *J. Histochem. Cytochem.* 37:729.
21. Kurokawa, I., A. Mayer-da-Silva, H. Gollnick, and C.E. Orfanos. 1988. Monoclonal antibody labeling for cytokeratins and filaggrin in the human pilosebaceous unit of normal, seborrhoeic and acne skin. 1988. *J. Invest. Dermatol.* 91:566.
22. Moore, G.P.M., B.A. Panaretto, and D. Robertson, 1981. Effects of epidermal growth factor on hair growth in the mouse. *J. Endocrinol.* 88:293.
23. Tam, J.P. 1985. Physiological effects of transforming growth factor α in the newborn mouse. *Science (Wash. DC).* 229:673.
24. Hollis, D.E., and R.E. Chapman. 1987. Apoptosis in wool follicles during mouse epidermal growth factor (mEGF)–induced catagen regression. *J. Invest. Dermatol.* 88:455.
25. Vassar, R., and E. Fuchs. 1991. Transgenic mice provide new insights into the role of TGF-α during epidermal development and differentiation. *Genes & Dev.* 5:714.
26. Du Cros, D.L. 1993. Fibroblast growth factor influences the development and cycling of murine hair follicles. *Dev. Biol.* 156:444.
27. Guo, L., Q.-C. Yu, and E. Fuchs. 1993. Targeting expression of keratinocyte growth factor to keratinocytes elicits striking changes in epithelial differentiation in transgenic mice. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:973.
28. Fuchs, E. 1990. Epidermal differentiation: the bare essentials. *J. Cell Biol.* 111:2807.
29. Wilke, M.S., B.M. Hsu, J.J. Wille, Jr., M.R. Pittelkow, and R.E. Scott. 1988. Biologic mechanisms for the regulation of normal human keratinocyte proliferation and differentiation. *Am. J. Pathol.* 131:171.
30. Régnier, M., P. Vaigot, M. Darmon, and M. Prunieras. 1986. Onset of epidermal differentiation in rapidly proliferating basal keratinocytes. *J. Invest. Dermatol.* 87:472.
31. Hertle, M.D., M.-D. Kubler, I.M. Leigh, and F.M. Watt. 1992. Aberrant integrin expression during epidermal wound healing and in psoriatic epidermis. *J. Clin. Invest.* 89:1892.
32. Grinnell, F. 1992. Wound repair, keratinocyte activation and integrin modulation. *J. Cell Sci.* 101:1.
33. Staiano-Coico, L., J.G. Krueger, J.S. Rubin, S. D'limi, V.P. Vallat, L. Valentino, T. Fahey, A. Hawes, G. Kingston, M.R. Madden, et al. 1993. Human keratinocyte growth factor (KGF) in a porcine model of epidermal wound healing. *J. Exp. Med.* 178:865.
34. Wenczak, B.A., J.B. Lynch, and L.B. Nanney. 1992. Epidermal growth factor receptor distribution in burn wounds. *J. Clin. Invest.* 90:2392.